THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N.Y. 10022
(212) 421-8885

JAN 2 2 1975

Application for Research Grant
(Use extra pages as needed)

Date: Jan. 13, 1975

1. Principal Investigator (give title and degrees): Simon H. Wender, Ph. D., Biochemistry, George L. Cross Research Professor

Co-Principal Investigator: Eddie C. Smith, Ph. D., Biochemistry, Professor

2. Institution & address: University of Oklahoma
Address: Department of Chemistry, 620 Parrington Oval, University of Oklahoma, Norman, Oklahoma, 73069

3. Department(s) where research willibe done or collaboration provided: Chemistry Department (Biochemistry Section)

4. Short title of study: Effect of Scopoletin and of Various "Stress" Conditions on the Activity of Certain Rat Lung Enzymes

5. Proposed starting date: July 1, 1975

6. Estimated time to complete: Three years

7. Brief description of specific research aims:

Our overall aim in the proposed research is to learn specific details of the actual interrelationships among scopoletin of cigarette smoke, various "stresses", and enzymes protecting against lung and/or other tissue damage, as well as other enzymes essential in normal metabolism.

Our group at the University of Oklahoma has recently discovered that scopoletin, esculetin, and chlorogenic acid (known components of cigarette smoke) inhibit the activity of control rat lung glucose-6-phosphate dehydrogenase, glutathione reductase and glutathione peroxidase. (See attached copy of a manuscript describing these findings as recently submitted for publication to LIFE SCIENCES.) These three enzymes are the ones that have been postulated by Chow and Tappel (1) for rat lung, and by Paglia and Valentine (2) and Beutler (3) for mammalian (including human) erythrocytes to comprise a protective mechanism against lipid peroxidation which might occur under various "stress" conditions such as exposure to the pollutants ozone, nitrogen dioxide and carbon tetrachloride; and chronic intake of excess ethyl alcohol.

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8. Brief statement of working hypothesis:

The previously mentioned work of Chow and Tappel, of Paglia and Valentine and of Beutler indicates that animals respond to an increase of lipid peroxides during certain "stress" condition by increasing the activity of glutathione peroxidase, which results in increased peroxide catabolism. This is the key feature of their proposed mechanisms. See accompanying figure I in endix for a mechanism summation by Chow and Tappel. Instead of peroxidation occurring during "stress" to produce tissue damage, harmless hydroxy fatty acids are formed by reduction of the lipid peroxides; these can then be metabolized via the normal β-oxidation pathway. The increases in the activity of glutathione reductase and glucose-6-phosphate dehydrogenase are also parts of the protective chain by providing the increased requirement for reduced glutathione and NADPH, respectively.

Since we have discovered that scopoletin, chlorogenic acid, and esculetin inhibit the three enzymes that comprise the above protective mechanism, we venture a preliminary working hypothesis that inhalation into the lung of scopoletin (and/or chlorogenic acid and esculetin) over an extended period of time may result in inhibition of this protective mechanism of figure I and possibly other protective mechanisms, so that these mechanism(s) may not be capable of protecting lung tissue adequately against the lipid peroxidation caused by certain "stress conditions". In healthy animals subjected to such a "stress", but not receiving scopoletin (cigarette smoke), the peroxidation possibly may be averted by the non-inhibited, actually increased, activity of the three enzymes.

9. Details of experimental design and procedures (append extra pages as necessary)

I. A. Comparison of the relative activity and isozyme composition of lung and also liver glucose-6-phosphate dehydrogenase, glutathione reductase, and glutathione peroxidase from rat controls vs. rats chronically under various "stress" conditions (not limited to those producing lipid peroxidation) such as listed below:

1. Rats nutritionally deficient in
   (a) Alpha-tocopherol (Vitamin E, a biological antioxidant aiding in protection against lipid peroxidation)
   (b) Methionine and cysteine (the latter, a constituent of glutathione)
   (c) Thiamine (vitamin B_1) and/or riboflavin (vitamin B_2) and/or niacinamide
   (d) Combinations of a, b, and c.

2. Rats supplied ethyl alcohol in relatively large amounts

3. Rats fed relatively high concentrations of fat (saturated vs. unsaturated)

4. Chronic exposure of rats to an atmosphere containing nitrogen dioxide or carbon tetrachloride

5. Rats subjected to chronic overcrowding

6. Rats subjected simultaneously to two or more of the above conditions

B. Comparison of the effect of scopoletin especially, but also chlorogenic acid and esculeti and also cigarette smoke particulate matter (containing these three compounds) on the activities of the above three enzymes in lung and liver homogenates from control vs. "stress" rats.

Isozymic forms of some enzymes may change qualitatively and/or quantitatively under varying "stress" conditions, and often individual isozymes are affected differently by the same effector compound. Hilf and coworkers (4) have reported recently that normal mammary glands in mice contained two isoenzymes of glucose-6-phosphate dehydrogenase, whereas a third, more rapidly migrating isoenzyme was found in significant levels only in the pre-neoplastic and neoplastic mammary tissues.

It is of interest to mention here - though we cannot state its precise relevance to animal metabolism as yet - that Wender and coworkers have shown that the amount of scopoletin, and especially of its glucoside scopolin, increases enormously in tobacco plants under many "stress" conditions to which the plant is subjected (5).

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II. A. Studies on the effect of scopoletin, etc. on the activity of other key animal enzymes, particularly oxidoreductases, and especially $NADP^+$ - utilizing dehydrogenases.

1. $NADP^+$ - specific isocitrate dehydrogenase

2. $NADP^+$ - specific malate dehydrogenase

3. Benzo - (α) - pyrene hydroxylase (using NADPH)

4. Various "mixed-function oxidases" (using NADPH)

5. Others, such as glyceraldehyde-3-phosphate dehydrogenase, alcohol dehydrogenase, lactate dehydrogenase, hydroxysteroid dehydrogenase, etc.

We venture the guess that probably some of these or other enzymes, in addition to the three above in I, may be involved in protective mechanisms against tissue damage. Possibly some of these may be affected to a substantial degree by scopoletin, thus also significantly affecting the ability of the animal tissue to be protected against damage during certain "stress" conditions. Some of these enzymes are also part of important metabolic pathways; for example, glucose-6-phosphate dehydrogenase of I is also a key enzyme at the beginning of the hexose monophosphate pathway, also called the pentose phosphate pathway. Inhibition of such enzymes by scopoletin may seriously modify normal metabolism. (For example, in some tissues, NADPH, as usually obtained in significant amounts from the glucose-6-phosphate dehydrogenase reaction, is required for steroid biosynthesis, including steroid sex hormones.) Ayers and Tierney (6) have reported that glucose-6-phosphate dehydrogenase activity was approximately twice as high in the supernatant of homogenized lungs of rats exposed to high PO_2 for 7-10 days and conclude that their "results are consistent with the hypothesis that the pentose phosphate pathway protects against high PO_2." Our Oklahoma findings would indicate that scopoletin is capable of inhibiting the activity of the first enzyme of this pathway, namely glucose-6-phosphate dehydrogenase, and hence passage through this pathway would be inhibited.

Our actual experimental approach in the study of the enzymes will be very similar to the established techniques, such as the methods of cell breakage and kinetic assays, currently used in our laboratory and many other laboratories. We have developed these necessary techniques and skills over the past several years with our studies of peroxidases and/or dehydrogenases from numerous sources including plant and human tissue cultures, plants, bacteria and mammalian tissues. Most are described in accompanying reprints of our recent publications.

We have previously found inhibition by scopoletin of the activity of enzymes 1 and 2 above when these dehydrogenases were obtained from tobacco tissue, but we have not yet tested the others.

Cross and coworkers (7) have pointed out the need of increasing NADPH formation from the glucose-6-phosphate dehydrogenase reaction in order to maintain the -SH levels in chronic ozone exposures. There are, of course, other enzymes besides glucose-6-phosphate dehydrogenase which are capable of supplying some NADPH.

Lange (8) has already reported an inhibition of purified rabbit muscle glyceraldehyde 3-phosphate dehydrogenase and yeast alcohol dehydrogenase by cigarette smoke.

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Aryl hydrocarbon hydroxylases are involved in oxidation of certain hydrocarbons, some of which may be carcinogenic.

III. Studies involving various combinations of I, II, and III. Results obtained in I and II will probably determine which ones are tried.

IV. Studies on the activity of glucose-6-phosphate dehydrogenase, glutathione reductase, glutathione peroxidase and other enzymes which may be found to be affected by scopoletin in II above in rats subjected to chronic exposure to cigarette smoke. The rats used will be controls and others under selected "stress" conditions.

We believe that step by step experimental progression of the research projected in I, II, and III will likely yield experimental results that will lead to the later designing of precise, pertinent experiments in IV which should give clearcut information on the actual in vivo situation regarding these enzymes and cigarette smoke.

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REFERENCES CITED IN PROPOSAL

1. Chow, C. K. and Tappel, A. L. 1972. "An Enzymatic Protection Mechanism Against Lipid Peroxidation Damage to Lungs of Ozone-Exposed Rats", LIPIDS 1 518.

2. Paglia, D. E. and Valentine, W. N. 1967. "Studies on the Quantitative and Qualitative Characterization of Erythrocyte Glutathione Peroxidase", J. LAB. and CLIN. MED. 70 158.

3. Beutler, Ernest 1972. "Drug-induced Anemia", FEDERATION PROC. 31 141.

4. Hilf, R., Rector, W. and Abraham, S. 1973. "A Glucose-6-Phosphate Dehydrogenase Isoenzyme Characteristic of Preneoplastic and Neoplastic Mouse Mammary Tissue", J. NATL. CANCER INST. 50 1395.

5. Wender, Simon H. 1970. "Effects of Some Environmental Stress Factors on Certain Phenolic Compounds in Tobacco", Chapter 1 in the book "Recent Advances in Phytochemistry", Vol. 3, Appleton-Century-Crofts, New York.

6. Ayers, L. and Tierney, D. 1971. "High PO_2 and the Pentose Pathway in Rat Lungs", CLINICAL RES. 19 190.

7. DeLucia, A. J., Hoque, P. M., Mustafa, M. and Cross, C. E. 1972. "Ozone Interaction with Rodent Lung: Effect on Sulfhydryls and Sulfhydryl Enzyme Activities", J. LAB. and CLIN. MED. 80 559.

8. Lange, R. 1961. "Inhibiting Effect of Tobacco Smoke on Some Crystalline Enzymes", SCIENCE 134 52.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Adequate research facilities for the proper prosecution of the investigation are available. The Chemistry Department occupied a new connecting addition in 1971. This new addition greatly enhanced our facilities for biochemical research since it contains a room devoted exclusively to the transfer and maintenance of tissue cultures, a large cold room with space for chromatography, adequate facilities and space for small animal research, a radioisotope room, a room to house our preparative ultracentrifuge and superspeed refrigerated centrifuge plus a general all purpose research room. Much of this research will, however, be carried out in the older part of the Chemistry building in two research laboratories which were completely remodeled in 1965. Among pertinent instruments available in the connecting Chemistry Department buildings are Varian Model 635K double beam recording UV-visible spectrophotometer, Beckman spectrometers, Model DK-1 and Model DU with special micro attachments a Hitachi-Perkin Elmer UV-visible spectrophotometer; Phipps and Bird cigarette smoking apparatus; Aminco-Bowman spectrophotofluorometer, complete with recorder and temperature control; Turner fluorimeter; Perkin-Elmer Model 21 double beam infrared spectrophotometer; Varian Associates Model A-60 analytical NMR spectrometer; Reco, GME, and Calbiochem fraction collectors; R&M Model 810R, and other gas chromatographs; numerous chromatocabs, and other paper, thin-layer and column chromatography facilities; Photovolt densitometer for scanning thin-layer chromatograms; numerous ultraviolet lamps, Blak-Ray Model B-100; Beckman scintillation counter; Beckman Model GP continuous flow paper electrophoresis system; Beckman amino acid analyzer; a Beckman preparative ultracentrifuge; and an Hitachi-Perkin-Elmer mass spectrometer.

A full-time instrument maker, electronics technician, and glassblower are available.

11. Additional facilities required:

None

12. Biographical sketches of investigator(s) and other professional personnel (append):

See attached sheets

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).
See attached sheets and reprints and reproductions of recently submitted manuscripts for publication.

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14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Simon H. Wender	15 → 25%	REDACTED
Eddie C. Smith	15 → 25%	REDACTED
Technical		
Post-doctorate (to be recruited)	100%	REDACTED
Graduate student research assistant (to be recruited)	50%	REDACTED
	Sub-Total for A	REDACTED
B. Consumable supplies (by major categories)		
Chemical, glassware, etc.		2,000
Animals (rats)		2,500
	Sub-Total for B	4,500.00
C. Other expenses (itemize)		
Fringe benefits for technical help (14,000 x 13.5%)		1,890
	Sub-Total for C	1,890
	Running Total of A + B + C	REDACTED
D. Permanent equipment (itemize)		
None		-0-
	Sub-Total for D	-0-
E. Indirect costs (15% of A+B+C)	E	3,058.50
	Total request	23,448.50

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	R	4,950.00	2,080.00	-0-	$3,364.35	25,793.35
Year 3	R	5,445.00	2,288.00	-0-	$3,700.95	28,373.95

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Purification and Characterization of Isoperoxidases from Tobacco	submitted to National Science Foundation	asked for $61,550 for the 2 years	July 1, 1975 to June 30, 1977

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Simon H. Wender

Signature Simon H. Wender Date Jan. 13, 197[illegible]

Telephone R - R
Area Code Number Extension

Checks payable to

University of Oklahoma

Mailing address for checks
University of Oklahoma
Office of Research Administration
1000 Asp Avenue, Room 314
Norman, Oklahoma 73069

Responsible officer of institution

Typed Name Raymond D. Daniels

Title Deputy Director, ORA

Signature Raymond D. Daniels Date 17 Jan 75

Telephone R R
Area Code Number Extension

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BIOGRAPHICAL SKETCHES

Principal Investigator: Dr. Simon H. Wender, George L. Cross Research Professor (Biochemistry) Chemistry Department, Norman, and Consultant Professor of Biochemistry, University of Oklahoma School of Medicine, Oklahoma City.

Date of Birth: R

Place of Birth: Dalton, Ga. (U.S.A.)

Present Citizenship: U.S.A.

Educational Experience:

Emory University, Ga., A.B. and M.S. in Chemistry, 1934 and 1935; U. of Minnesota, Ph.D., Biochemistry, 1938.

University of Oklahoma, George L. Cross Research Professor of Biochemistry (a distinguished Professorship) since 1953; prior to that full Professor, 1948-1953; and Associate Professor, 1946-48.

Consultant Professor of Biochemistry, University of Oklahoma School of Medicine, 1968 —.

University of Kentucky, Instructor, then Assistant Professor, 1942-1946. Texas Agricultural Experiment Station, Associate Chemist, 1939-1942.

Argonne National Laboratory, Div. of Biol. and Med. Research, summers 1953 and 1958. (Visiting research associate 1954-1964).

Fields of Present Major Scientific Interest:

Biochemistry and Chemistry of Phenolic Compounds in Plants; Chromatography; and Plant Oxidoreductases.

Other Information:

Dr. Wender has been a member of the Board of Directors of Oak Ridge Associated Universities, Inc., (1961-1967) and past chairman of the Oak Ridge Council. He is the present Councilor from the University of Oklahoma to ORAU.

Dr. Wender is a member of Phi Beta Kappa, Sigma Xi (former President, Okla. Chapter), Phi Lambda Upsilon, Phi Sigma, Alpha Chi Sigma, American Chemical Society (fomer Chairman, Okla. Section and also Lexington, Ky. Section), American Society of Biological Chemists, American Association for the Advancement of Science (Fellow), American Society of Plant Physiologists, Society for Experimental Biology and Medicine, Oklahoma Academy of Science (Fellow), Phytochemical Society of North America (first International President, 1961-1962), Scandinavian Society for Plant Physiology, and American Institute of Biological Sciences.

During the fall 1966-1967 semester, Dr. Wender was on sabbatical leave doing full-time research on plant phenols and tobacco tissue cultured in vitro, with Dr. Folke Skoog, Botany Department, University of Wisconsin.

During the spring 1974 semester, Dr. Wender was on sabbatical leave doing research on plant phenols with Dr. Eric Conn, Department of Biochemistry and Biophysics, University of California at Davis.

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Co-Principal Investigator: Dr. Eddie Carol Smith

Born:

Height: REDACTED REDACTED

Married to:

REDACTED

Children:

Education:

B.S. in Chemistry: University of Kentucky May 1959
Ph.D. in Biochemistry: Iowa State University July 1963

Professional Experience:

Research Associate: Iowa State University under Dr. David Metzler July 1963-December 1963
Post-Doctoral Scholar: UCLA under Dr. Daniel Atkinson January 1964-September 1965
Assistant Professor: Oklahoma University September 1965-September 1969
Associate Professor: Oklahoma University September 1969-September 1974
Full Professor: Oklahoma University September 1974

Professional Societies:

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PUBLICATIONS OF CO-PRINCIPAL INVESTIGATORS

Dr. Wender is author or co-author of more than 170 research publications. These are concerned primarily with the biochemistry and chemistry of phenolic compounds in various fruits, vegetables, and especially in tobacco and cigarette smoke. In the past four years, he has collaborated with Dr. Eddie C. Smith, an experienced enzymologist, on various enzymes whose activity might be affected by phenolic compounds.

The most recent plus selected, pertinent previous publications by each co-principal investigator, and jointly, are listed below:

"The Effect of Certain Naturally Occurring Compounds on the Activity of Rat Lung Glucose-6-Phosphate Dehydrogenase, Glutathione Reductase and Glutathione Peroxidase", by Pickering, Jerry W., Wender, Simon H. and Smith, Eddie C., LIFE SCIENCES, submitted 1975.

"Substrate Specificities and Molecular Weights of Four Isoperoxidases from Tobacco Tissue Culture", by Powell, B. L., Pickering, J. W., Wender, S. H. and Smith, E. C., PHYTOCHEMISTRY, submitted Nov. 1974.

"Effect of Complete Darkness and of Intense Light on Isoperoxidases in Nicotiana Tabacum Tissue", by Leu, S-L. K., Wender, S. H. and Smith, E. C. PHYTOCHEMISTRY, submitted Nov. 1974.

"Scopoletin: Kinetic Nature of Isoperoxidase A_3 Catalyzed Oxidation and Its Possible Relation to the Physiological Action of this Naturally-occurring Growth Effector", by Reigh, D. L., Wender, S. H. and Smith, E. C., PHYSIOLOGIA PLANTARUM, IN PRESS.

"Phenolic Inhibition of Isoperoxidase A_3", by Reigh, D. L., Wender, S. H. and Smith, E. C., TOBACCO SCIENCE 18 88 (1974).

"Scopoletin: A Substrate for an Isoperoxidase from Nicotiana Tabacum Tissue Culture W-38", by Reigh, D. L., Wender, S. H. and Smith, E. C., PHYTOCHEMISTRY 12 1265 (1973).

"Quantitative Studies of Scopoletin in Cigarette Smoke and Tobacco", by Yang, C-H., Nakagawa, Y. and Wender, S., ANALYTICAL CHEMISTRY 30 2041 (1958).

"Effects of Some Environmental Stress Factors on Certain Phenolic Compounds in Tobacco", by Wender, Simon H., Chapter 1 in the book "Recent Advances in Phytochemistry", Vol. 3, Appleton-Century-Crofts, New York, 1970.

"Evidence for Different Glyceraldehyde-3-Phosphate Dehydrogenases in WI-38 and SV 40-Transformed WI-38", by Dunaway, G. A., Jr., and Smith, E. C., INT. J. BIOCHEM. 4 245 (1973).

"A Comparative Study of Some of the Enzymes Involved in Glucose Metabolism of Human Diploid and SV-40 Transformed Diploid Human Cells", by Dunaway, G. A., Jr., and Smith, E. C., CANCER RESEARCH 31 1418 (1971).

"Glucose-6-Phosphate Dehydrogenase from Tobacco Tissue Culture WR-132. I. Inhibition by Scopoletin and Scopolin", by Kajinami, S., Wender, S. H. and Smith, E. C., PHYTOCHEMISTRY 10 1501 (1971).

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APPENDIX

Effect of Scopoletin and of Various "Stress Conditions on the Activity of Certain Rat Lung Enzymes

by S. H. Wender and E. C. Smith

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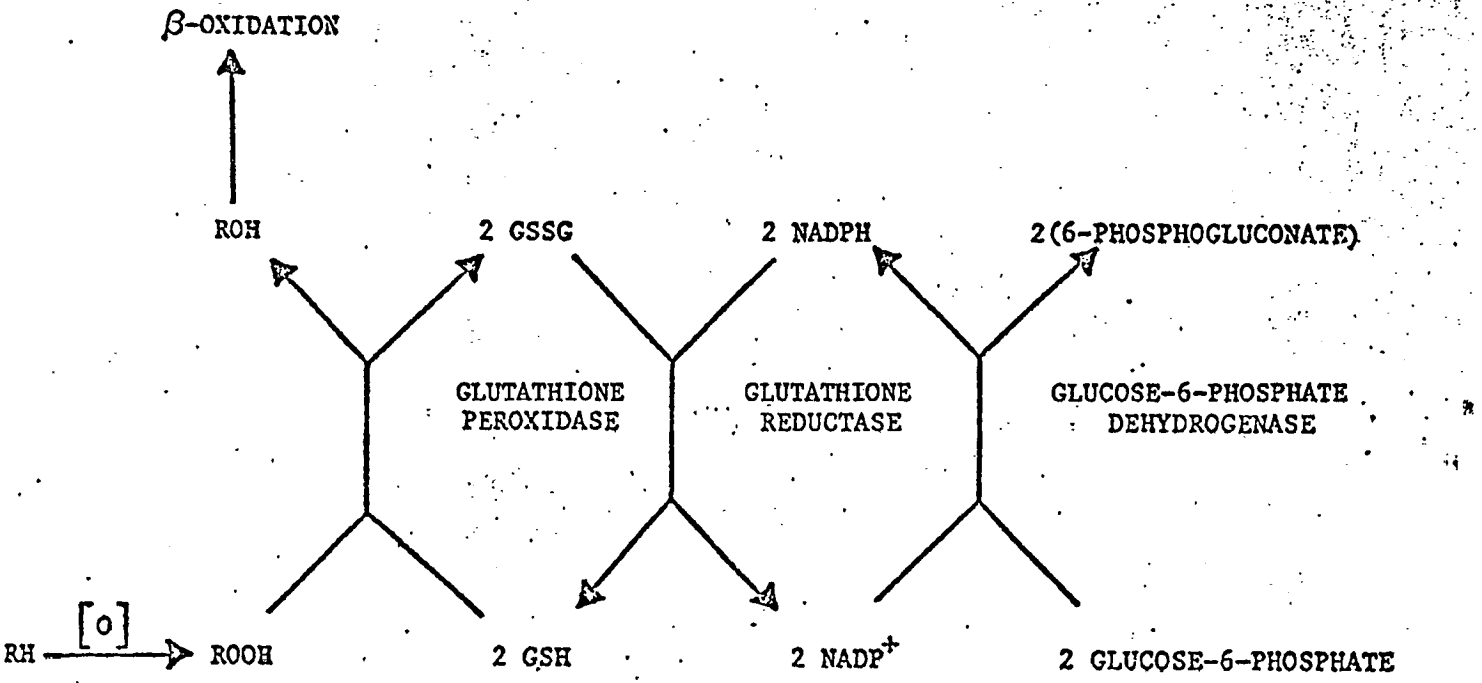
Figure 1



SCHEME OF LIPID PEROXIDE METABOLISM. RH, polyunsaturated fatty acid; ROOH, fatty acid hydroperoxide; ROH, hydroxy fatty acid; GSH, reduced glutathione; GSSG, oxidized glutathione.

Chow, C. K. and Tappel, A. L., Lipids, 1, 518-524. (1972).

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[redacted]

manuscript submitted this 1978 to Life Sciences

THE EFFECT OF CERTAIN NATURALLY OCCURRING COMPOUNDS ON THE ACTIVITY OF RAT LUNG GLUCOSE-6-PHOSPHATE DEHYDROGENASE, GLUTATHIONE REDUCTASE AND GLUTATHIONE PEROXIDASE

Jerry W. Pickering, Simon H. Wender and Eddie C. Smith

Biochemistry Section, Chemistry Department, University of Oklahoma, Norman, Oklahoma 73069, U.S.A.

Received:_____

Summary

Nicotine and a number of phenolic compounds known to be components of either cigarette smoke, or tobacco, or both, were tested as possible effectors of the activities of glutathione peroxidase, glutathione reductase and glucose-6-phosphate dehydrogenase in rat lung homogenates. At a concentration of 0.4 mM, five of these compounds inhibited (up to 90% in some cases) all three of the above enzymes. Other workers have previously proposed that these same three enzymes comprise a protective mechanism in lung against damage by lipid peroxidation.

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Introduction

Glutathione has been shown to reduce potentially damaging lipid peroxides in rat liver homogenates (1). Glutathione peroxidase catalyzed the involved reaction (2). DeLucia et al. (3) have reported that oxidative damage to sulfhydryl (SH) groups in rat lung by prolonged exposure to ozone is counteracted by an increase in the activity of glucose-6-phosphate (G6P) dehydrogenase and a

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corresponding increase in the levels of reduced nicotinamide adenine dinucleotide phosphate (NADPH). Chow and Tappel (4) have shown that the activities of glutathione peroxidase, glutathione reductase and G6P dehydrogenase are increased significantly in lung homogenates of rats exposed to ozone. They proposed that these enzymes may comprise a protective mechanism to detoxify lipid peroxides resulting from exposure to ozone and other air oxidants. In the present paper, we report the effect of certain individual compounds present in cigarette smoke, or tobacco, or both, upon the activity of the three enzymes included in the above protective mechanism against lipid peroxidation.

Methods and Materials

Enzyme preparation: Lung homogenates were prepared according to a modification of the procedure of Chow and Tappel (4). Mature rats which had been fed a normal diet were sacrificed. Lungs were removed immediately and washed in isotonic KCl. A 20% suspension of the rat lungs in 0.25 M sucrose containing 1 mM EDTA was homogenized at 6,000 r.p.m. for 10 min. in a Sorvall Omnimixer. The homogenate was centrifuged first at 100 x g for 10 min. and then at 105,000 x g for 90 min. to yield a clear red supernatant used for enzyme assays.

Enzyme assays: The assay procedure for glutathione reductase was based upon that of Racker (5). The assay solution contained 0.05 M phosphate buffer (pH 7.6), 0.1 M NADPH, 0.1% BSA and 0.2% oxidized glutathione in a total volume of 3.0 ml. The enzyme reaction was initiated by the addition of 0.1

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ml of the enzyme preparation.

Glucose-6-phosphate dehydrogenase activity was determined by the method of Brown and Wray (6), with the exception that Tris buffer (pH 7.5) was utilized according to the procedure reported for erythrocyte G6P dehydrogenase (7). The assay solution contained 0.02 M Tris-HCl buffer (pH 7.5), 0.3 mM $NADP^+$, 5 mM D-glucose-6-phosphate and 5 mM $MgCl_2$ in a total volume of 3.0 ml. The reaction was initiated by addition of 0.2 or 0.3 ml of the enzyme preparation.

The assay procedure for glutathione peroxidase was that of Paglia and Valentine (8). Commercial glutathione reductase (Sigma) was used in this assay. All effector compounds were prepared in 2% ethanol. An equivalent volume of 2% ethanol was added in control assays.

Results

The effect upon the activities of rat lung glutathione peroxidase, glutathione reductase and G6P dehydrogenase of nicotine and certain phenolic compounds known to be components of either cigarette smoke, or tobacco, or both, is shown in Table I. At a concentration of 0.4 mM, five of these phenolic compounds, namely scopoletin, scopolin, esculetin, esculin and chlorogenic acid, markedly inhibited the activity of glutathione reductase and of G6P dehydrogenase and also substantially inhibited the glutathione peroxidase assay system. None of the other compounds tested exhibited any significant effect upon the activity of any of the three enzymes. However, since the coupled enzyme assay previously used by Paglia and Valentine (8) and which requires glutathione reductase, was used in the glutathione peroxidase assay and since the purified commercial

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Table I

Effects of Phenolic Compounds and Nicotine on Activity of Three Enzymes from Rat Lung

0.4 mM Effector	Glutathione Peroxidase	Glutathione Reductase	Glucose-6-phosphate Dehydrogenase
None	100	100	100
Caffeic acid	100	102	99
Chlorogenic acid	24	33	13
p-Coumaric acid	102	102	100
Esculetin	12	45	68
Esculin	16	45	49
Ferulic acid	101	100	100
Nicotine	102	102	100
Salicylic acid	102	96	100
Scopoletin	14	37	43
Scopolin	17	55	27
Vanillic acid	100	102	100

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glutathione reductase used in the assay system displayed the same pattern of inhibition as the rat lung enzyme, it is not possible at present to determine how much of the observed inhibition can be attributed to the inhibition of glutathione peroxidase. It is speculated that a significant amount of the inhibition is due to glutathione peroxidase because of the excess quantities of glutathione reductase used in the assay; however, quantitative measurements await the use of a direct assay for glutathione peroxidase.

Discussion

Of those phenolic compounds which we have found to have an inhibitory effect on the enzyme systems studied, scopoletin, esculetin, and chlorogenic acid have been previously shown to be present in cigarette smoke. Scopoletin is by far the most abundant of the three in the smoke. In 1958, scopoletin was identified in the mainstream smoke of all 29 different brands of cigarettes tested, some of which had filters (9). The amount of scopoletin present in smoke for each 1 g of tobacco originally present in the smoked cigarette ranged from 10.3 to 27.4 µg. Chlorogenic acid (10) and esculetin (11) have also been identified in trace amounts in cigarette smoke. Scopolin and esculin, which are glucosides of scopoletin and esculetin, respectively, have not been found in cigarette smoke, although they have been reported to be present in tobacco plants (12).

The smoke from cigarettes has been shown by several groups of research workers to inhibit some dehydrogenases. Sato and coworkers (13) have reported that inhibition of succinic dehydrogenase by cigarette smoke was eliminated

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by cysteine or glutathione. Lange (14) reported an inhibition of purified rabbit muscle glyceraldehyde-3-phosphate dehydrogenase and yeast alcohol dehydrogenase by cigarette smoke. The observed inhibition of both of these enzymes was partially overcome by prior inhalation of the smoke. Lange suggested that this inhibitory effect of cigarette smoke might be the result of oxidation of SH groups by peroxides which might be present in the smoke. Subsequently (15), peroxides were identified in cigarette smoke.

Using specific effectors and enzyme system assays, we have found that the phenolic lactone scopoletin, a significant component of cigarette smoke, inhibits glutathione reductase, G6P dehydrogenase, and possibly glutathione peroxidase from rat lungs. These enzymes are the very ones that have been postulated by Chow and Tappel (4) to comprise a protective mechanism against lipid peroxidation in mammals.

Acknowledgement

This research was supported in part by a grant from the National Institutes of Health.

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References

1. B. O. CHRISTOPHERSEN, Biochem. J. 100, 95 (1960).

2. B. O. CHRISTOPHERSEN, Biochim. Biophys. Acta 164, 35 (1968).

3. A. J. DeLUCIA, P. M. HOQUE, M. G. MUSTAFA and C. E. CROSS, J. Lab. and Clin. Med. 80, 599 (1972).

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10035465 41

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